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The systematic position of *Phaeocryptopus gaeumannii*

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Abstract: *Phaeocryptopus gaeumannii*, causal agent of the Douglas-fir foliar disease Swiss needle cast, is the only known pathogenic species of the genus. Current classifications place *Phaeocryptopus* in the Venturiaceae (Pleosporales), typified by the apple-scab pathogen *Venturia inaequalis*. All core members of this family have hyphomycetous anamorphs. We sought to confirm these relationships by means of phylogenetic analyses of the small (SSU) and large (LSU) subunits and internal transcribed spacer (ITS) region of nuclear ribosomal gene sequences (nrDNA). Analyses indicated that both the genus *Phaeocryptopus* and the family Venturiaceae, as currently defined, are unnatural groups. *Phaeocryptopus nudus*, type of the genus, is aligned in the Dothioraceae (Dothideales) and *P. gaeumannii* in the Mycosphaerellaceae (Capnodiales) near species of *Mycosphaerella* and *Rasutoria*. Core representatives of Venturiaceae formed an unambiguous clade but ordinal placement was unresolved. The family apparently is not included in the Pleosporales, Dothideales, Myriangiales or Capnodiales. Coelomycetous *Rhizosphaera* form-species are accepted generally as anamorphic states of *Phaeocryptopus*, however the relationship never has been established conclusively. Species of *Rhizosphaera* are closely related to *P. nudus* but not to *P. gaeumannii*, supporting an anamorph-teleomorph connection between *Rhizosphaera* and *Phaeocryptopus* and providing further evidence that *P. gaeumanii* is not congeneric with *P. nudus*.

Key words: Capnodiales, Dothorioraceae, loculoascomycetes, Mycosphaerellaceae, nrDNA, phylogenetics, Swiss needle cast, Venturiaceae

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INTRODUCTION

Swiss needle cast first was described by Gäumann (1930) as causing serious defoliation in Douglas-fir (*Pseudotsuga menziesii* [Mirb.] Franco) plantations in Europe. Although he was uncertain of the complicated taxonomy of the fungus, he stated ''So far as the observations presented are concerned, our fungus…is an ascomycete belonging to the genus *Adelopus*, according to our knowledge, a single species is known in this genus, namely *Adelopus balsamicola* (Peck) Theiss''. Theissen (1917) used the name *Adelopus balsamicola* because Saccardo (1915) considered *Asterella nuda* (Peck) Sacc. a synonym of *Meliola balsamicola* Peck (=*Dimerosporium balsamicola* [Peck] Ell. & Ev.) Consequently *Asterina nuda* Peck frequently has been confused in the literature with *Meliola balsamicola* Peck (now *Dimerium balsamicola* [Peck] Shoemaker [1965]). Hahn (1947) corrected the confusion concerning the two species, *M. balsamicola* and *Asterina nuda*, confirming that Peck had intended to erect the two distinctly different species in different genera. The combination *Adelopus nudus* (Peck) Theiss. resulted from a new combination attributed to Theissen by von Höhnel (1918) and perpetuated by Petrak (1925) and Rohde (1936, 1937). Peck (1885) originally described the fungus as *Asterina nuda* Peck on needles of *Abies balsamea* (L.) Mill. in New York. Because Peck's description of this fungus did not match the description of *Asterina* Lév., Saccardo transferred it to *Asterella nuda* (Peck) Sacc. in 1891. Theissen (1914) determined that the fungus belonged neither in *Asterina* nor *Asterella* and proposed the new genus *Cryptopus*, typified by *Cryptopus nudus* (Peck) Theiss. Three years later Theissen and Sydow discovered that *Cryptopus* Lindl. previously had been erected as a member of the Orchidaceae and proposed *Adelopus* Theiss. (1917) as a replacement.

After studying new (not type) materials of *Phaeocryptopus abietis* Naumov (1915), Petrak (1938) determined that *Adelopus nudus* (Peck) Theiss. was identical to *P. abietis* Naumov, originally found on *Abies sibirica* in Perm, Ural. This resulted in the combination *Phaeocryptopus nudus* (Peck) Petrak. However both Hahn (1947), openly and Wilson and Waldie (1928) by implication questioned the synonymy. Petrak (1938) also considered the coelomycetous, mitosporic genus *Rhizosphaera* to be the anamorph of *Phaeocryptopus*. Although this connection was disputed by Rohde (1936), who presented

experimental evidence to the contrary, Petrak dismissed Rohde's observations, stressing ecological and morphological similarities. *Rhizosphaera* has been accepted generally as anamorphic *Phaeocryptopus* and is listed as such in the 8th and 9th editions of Ainsworth and Bisby's Dictionary of the Fungi (Hawksworth et al 1995, Kirk et al 2001).

The causal agent of Swiss needle cast first was named *Adelopus gaeumannii* Rohde (Rohde 1936, 1937). Petrak (1938) proposed the new combination *Phaeocryptopus gaeumannii* (Rhode) Petrak. He included *Adelopus balsamicola* (Peck) Theiss. f. *douglasii* (Steiner 1937) as a synonym and did not accept Steiner's belief that the fungus found on *Pseudotsuga* was merely a form of the species on *Abies*.

The first reference to familial placement of *Phaeocryptopus* was Theissen's initial placement of '*Cryptopus' nudus* in the Capnodiaceae (1914), but he later placed it in the Chaetothyriaceae as *Adelopus nudus* (1917). Petrak (1938) regarded both families as artificial and recommended their re-evaluation. Müller and von Arx (1950) erected Venturiaceae and included *Phaeocryptopus* within it. Barr (1968) retained *Phaeocryptopus* in Venturiaceae in a complete treatment of the family in North America. At that time she recognized 13 genera with 80 species, which she later modified slightly (Barr 1987b). The apple scab pathogen *Venturia inaequalis* was designated as type of the family. Because of the economic importance of apple scab disease, the pathology and epidemiology of *V. inaequalis* has been well studied. The putative familial relationship between *V. inaequalis* and *P. gaeumannii* has prompted suggestions that control measures for a new epidemic of Swiss needle cast might be adapted from apple scab management (Hansen et al 2000).

Traditional classifications have placed Venturiaceae in Pleosporales, a complex order characterized by J-, fissitunicate asci, asymmetric ascospores, asci in a basal layer, paraphysoids or pseudoparaphyses, and a pseudoparenchymatous peridium (Barr 1987b). Ascomycetous fungi with fissitunicately discharging asci that are borne in locules within ascostromatic ascomata (pseudothecia) have been variously grouped depending on which characters have been emphasized. Nannfeldt's (1932) Ascoloculares, Luttrell's (1951) Bitunicatae and Luttrell's (1973) Loculoascomycetes have proposed different arrangements of 3–11 orders (Müller and von Arx 1962; Luttrell 1973; von Arx and Müller 1975; Barr 1979, 1987b). Von Arx and Müller (1975) placed all bitunicate ascomycetes in the Dothideales, in an admittedly unnatural classification.

Uncertainty regarding higher order classification of ascomycetes has led to abandonment of ranks above order (Hawksworth et al 1995) until relationships are better resolved. In the classification proposed by Eriksson (1982) class and subclass ranks were replaced by descriptive names of ascomatal types. New names based on type species more recently have been proposed for monophyletic groups recovered largely from phylogenetic analyses of DNA sequence data (Eriksson and Winka 1997, 1998; Eriksson 1999). The 11 core orders of loculoascomycetes currently are classified provisionally in Dothideomycetidae, although the limitations of molecular sequence data for this large and varied group are recognized (Kirk et al 2001). Single gene phylogentic analyses so far have failed to unequivocally establish relationships among the loculoascomycetes (Berbee 1996, Liew et al 2000, Lumbsch et al 2000, Reynolds 1998, Silva-Hanlin and Hanlin 1999, Winka et al 1998). Multigene approaches are proving more informative. Higher order relationships within the Dothideomycetes recently have been clarified by Schoch et al (2006), who recognized two subclasses and six orders, including monophyletic Pleosporales and Dothideales. Placement of Venturiaceae was unresolved because no exemplars of the family were included. Species of *Venturia*, *Spilocea* and *Metacoleroa*, genera traditionally classified in Venturiaceae, formed a monophyletic group in the analysis of Kruys et al (2006), based on nLSU, nSSU and mtSSU sequence data.

The purpose of this study was to use small (SSU) and large (LSU) subunits and internal transcribed spacer region (ITS) of nuclear ribosomal DNA (nrDNA) sequences to estimate phylogenies to test several hypotheses. Those explicitly tested are: (i) *Phaeocryptopus nudus* and *P. gaeumannii* are congeneric, (ii) *P. gaeumannii* is closely related to *Venturia inaequalis*, (iii) *P. nudus* is closely related to *V. inaequalis*, (iv) *Rhizosphaera* species are closely related to *P. nudus*, (v) *Rhizosphaera* species are closely related to *P. gaeumannii*. Important corollary observations not specifically tested included evaluating monophyly of the Venturiaceae and other groups of the loculoascomycetous fungi.

MATERIALS AND METHODS

Fungal cultures.—Cultures used in this study (TABLE I) were obtained either from culture collections or isolated from host tissue. Single-ascospore isolates were obtained for *Apiosporina collinsii*, *Dibotryon morbosum*, *Metacoleroa dickiei*, *P. gaeumannii*, *P. nudus*, *Rasutoria pseudotsugae*, *R. tsugae*, *Sthughesia juniperi* and a *Stomiopeltis* sp. by suspending foliage bearing pseudothecia (attached to the underside of the Petri dish lids) above Petri dishes containing water agar (2%). Samples were incubated in a moist chamber at 17 C and individual ascospores removed from the agar surface with a heat-drawn Pasteur pipette. Identical methods were used to obtain single-conidium

TABLE I. Taxa and GenBank accession numbers included in phylogenetic analyses of nuclear rDNA small (SSU) and large (LSU) subunits and internal transcribed spacer (ITS). Classifications mainly follow that of Eriksson (2006) and Kirk et al (2001).

Class/order/family	Species	GenBank accession no.[a] SSU	LSU	ITS
Arthoniomycetes (Outgroup)				
Arthoniales				
Roccellaceae	*Dendrographa leucophaea f. minor*	AF279381	AF279382	—
	Roccella fuciformis	AY584678	AY584654	—
Dothideomycetes				
Capnodiales				
Capnodiaceae	*Capnodium coffeae*	DQ247808	DQ247800	DQ491515
	Capnodium salicinum	DQ677997	DQ678050	AJ244240
	Scorias spongiosa	DQ678024	DQ678075	—
anamorphic	*Capnobotryella renispora*	—	—	AY220612
Metacapnodiaceae [c]	***Sthughesia juniperi*** [c]	**EF114734**	**EF114709**	**EF114689**
Dothideales				
Dothideaceae	*Dothidea insculpta*	DQ247810	DQ247802	AF027764
	Dothidea sambuci	AY544722	AY544681	—
	Stylodothis puccinioides	AY016353	AY004342	—
Dothioraceae	*Delphinella strobiligena*	DQ471029	DQ470977	—
	Dothiora cannabinae	DQ479933	DQ470984	—
	Dothiora rhamni—alpinae	—	—	AJ244245
	Plowrightia abietis	**EF114727**	**EF114703**	**—**
	Sydowia polyspora	DQ678005	DQ678058	—
Hysteriales				
Hysteriaceae	*Hysterium pulicare*	DQ678002	DQ678055	—
	Hysteropatella clavispora	DQ678006	AY541493	—
Myriangiales				
Elsinoaceae	*Elsinoe centrolobi*	DQ678041	DQ678094	—
	Elsinoe phaseoli	DQ678042	DQ678095	—
	Elsinoe veneta	DQ767651	DQ767658	—
Myriangiaceae	*Myriangium duriaei*	AY016347	DQ678059	—
Pleosporales				
Delitschiaceae	*Delitschia winteri*	DQ678026	DQ678077	—
Lophiostomataceae	*Herpotrichia juniperi*	DQ678029	DQ678080	—
	Lophiostoma crenatum	DQ678017	DQ678069	—
Massariaceae	*Massaria platani*	DQ678013	DQ678065	—
Melanommataceae	*Bimuria novae—zelandiae*	AY016338	AY016356	—
	Trematosphaeria pertusa	DQ678020	DQ678072	—
Montagnulaceae	*Montagnula opulenta*	AF164370	DQ678086	—
Phaeosphaeriaceae	*Leptosphaeria maculans*	DQ470993	DQ470946	—
	Ophiosphaerella herpotricha	DQ678010	DQ678062	—
	Phaeosphaeria avenaria	AY544725	AY544684	—
anamorphic	*Coniothyrium obiones*	DQ678001	DQ678054	—
Phaeotrichaceae	*Phaeotrichum benjaminii*	AY016348	AY004340	—
Pleomassariaceae	*Pleomassaria siparia*	DQ678027	DQ678078	—
Pleosporaceae	*Cochliobolus heterostrophus*	AY544727	AY544645	—
	Pleospora herbarum	DQ767648	DQ678049	—
	Pyrenophora tritici—repentis	AY544716	AY544672	—
Sporormiaceae	*Preussia terricola*	AY544726	AY544686	—
Teichosporaceae	*Byssothecium circinans*	AY016339	AY016357	—
Venturiaceae	***Apiosporina collinsii*** [b]	**—**	**EF114692**	**—**
	Dibotryon morbosum	**EF114718**	**EF114694**	**—**
	Metacoleroa dickiei	**EF114719**	**EF114695**	**—**
	Phaeocryptopus gaeumannii [b]	**EF114722**	**EF114698**	**EF114685**
	Phaeocryptopus nudus [b]	**EF114724**	**EF114700**	**—**
	Platychora ulmi	**EF114726**	**EF114702**	**—**

TABLE I. Continued

Class/order/family	Species	GenBank accession no.[a] SSU	LSU	ITS
	Protoventuria barriae	**EF114728**	—	—
	Venturia asperata	**EF114736**	**EF114711**	—
	Venturia inaequalis [b]	**EF114737**	**EF114712**	—
	Venturia pyrina [b]	**EF114739**	**EF114714**	—
	Xenomeris raetica	**EF114741**	**EF114716**	**EF114690**
anamorphic	***Rhizosphaera kalkhoffii***	**EF114731**	**EF114706**	—
	Rhizosphaera oudemansii	**EF114732**	**EF114707**	—
	Rhizosphaera pini	**EF114733**	**EF114708**	—
Dothidiomycetes et Chaetothyriomycetes *incertae sedis*				
Botryosphaeriaceae	*Botryosphaeria dothidea*	DQ677998	DQ678051	—
	Botryosphaeria ribis	DQ678000	DQ678053	—
	Guignardia bidwellii	DQ678034	DQ678085	—
Cucurbitariaceae	*Cucurbitaria elongata*	DQ678009	DQ678061	—
Didymosphaeriaceae	*Phaeodothis winteri*	DQ678021	DQ678073	—
	Verruculina enalia	DQ678028	DQ678079	—
Euantennariaceae	***Rasutoria pseudotsugae***	**EF114729v**	**EF114704**	**EF114687**
	Rasutoria tsugae	**EF114730**	**EF114705**	**EF114688**
Micropeltideaceae	***Stomiopeltis sp.***	**EF114735**	**EF114710**	—
Mycosphaerellaceae	*Davidiella macrospora*	—	—	AF362049
	Davidiella tassiana	DQ678022	DQ678074	—
	Discosphaerina fagi	AY016342	AY016359	—
	Mycosphaerella africana	—	—	AF309602
	Mycosphaerella brassicicola	—	—	AY152557
	Mycosphaerella citri	—	—	AF181703
	Mycosphaerella colombiensis	—	—	AF309612
	Mycosphaerella cruenta	—	—	AY266153
	Mycosphaerella cryptica	—	—	AF309623
	Mycosphaerella crystallina	—	—	AF309611
	Mycosphaerella ellipsoidea	—	—	AF309592
	Mycosphaerella fijiensis	—	—	AF297225
	Mycosphaerella fijiensis	—	—	AF297234
	Mycosphaerella flexuosa	—	—	AF309603
	Mycosphaerella fragariae [b]	**EF114720**	**EF114696**	AF173312
	Mycosphaerella graminicola	DQ678033	DQ678084	AF181694
	Mycosphaerella heimii	—	—	AF309606
	Mycosphaerella heimioides	—	—	AF309609
	Mycosphaerella irregulariramosa	—	—	AF309608
	Mycosphaerella juvenis	—	—	AF309605
	Mycosphaerella keniensis	—	—	AF309601
	Mycosphaerella marasasii	—	—	AF309591
	Mycosphaerella marksii	—	—	AY725556
	Mycosphaerella molleriana	—	—	AF309620
	Mycosphaerella musicola	—	—	AF181706
	Mycosphaerella nubilosa	—	—	AF309618
	Mycosphaerella parkii	—	—	AF173311
	Mycosphaerella pini [b]	**EF114721**	**EF114697**	AF211197
	Mycosphaerella punctiformis	DQ471017	DQ470968	AY490763
	Mycosphaerella suttonii	—	—	AF309621
	Mycosphaerella syzygii	—	—	AF309610
	Mycosphaerella tasmaniensis	—	—	AF173307
	Mycosphaerella walkeri	—	—	AY045502

TABLE I. Continued

Class/order/family	Species	GenBank accession no.[a] SSU	LSU	ITS
anamorphic	*Cercospora nicotianae*	—	—	AY266159
	Cladosporium cladosporioides	**EF114717**	**EF114693**	—
Piedraiaceae	*Piedraia hortae*	AY016349	AY016366	—
Tubeufiaceae	*Tubeufia cerea*	DQ471034	DQ470982	—
anamorphic	*Helicomyces roseus*	DQ678032	DQ678083	—
Dothideomycetes *incertae sedis*				
	Didymella cucurbitacearum	AY293779	AY293792	—
	Tyrannosorus pinicola	DQ471025	DQ470974	—
anamorphic	*Ramichloridium cerophilum*	—	—	AF050286
	Trimmatostroma abietis	DQ678040	DQ678092	—

[a] New sequences reported in this study in bold.

[b] Additional sequences obtained for this study for quality control but not used in phylogenetic analyses. *Apiosporina collinsii*: **EF114691** (ITS); *Mycosphaerella fragariae*: **EF114683** (ITS); *Mycosphaerella pini*: **EF114684** (ITS); *Phaeocryptopus gaeumannii*: **EF114723** (SSU), **EF114699** (LSU), **EF114686** (ITS); *Phaeocryptopus nudus*: **EF114725** (SSU), **EF114701** (LSU); *Venturia inaequalis*: **EF114738** (SSU), **EF114713** (LSU); *Venturia pyrina*: **EF114740** (SSU), **EF114715** (LSU).

[c] Classification of Barr (1987a).

isolates of *Rhizosphaera oudemansii* and *R. pini*. *Cladosporium cladosporioides*, *Mycosphaerella fragariae*, *Venturia inaequalis* and *V. pyrina* were isolated from host tissue surface-sterilized in 95% ethanol for 60 s and 2% NaOCl for 5 min. Representatives of *Mycosphaerella pini*, *P. gaeumannii*, *P. nudus*, *Platychora ulmi*, *Plowrightia abietis*, *Protoventuria barriae*, *Rhizosphaera kalkhoffii*, *Venturia asperata*, *V. inaequalis*, *V. pyrina* and *Xenomeris raetica* were obtained from the American Type Culture Collection (ATCC) or Centraalbureau voor Schimmelcultures (CBS). Additional information regarding strain identification number and location is available on GenBank under the accession numbers provided (TABLE I). All fungal cultures were maintained on potato-dextrose agar (Difco Laboratories, Detroit, Michigan).

DNA isolation and sequencing.—Fungal cultures were prepared for extraction by scraping about 30 mg mycelium from the agar surface. Collected mycelium was placed into 2 mL microfuge tubes with 1 mm zirconia/silica beads (Biospec Products, Bartlesville, Oklahoma) and 1 mL CTAB extraction buffer (2% CTAB [cetyltrimethylammonium bromide], 100 mM Tris, pH 8.0, 20 mM Na_2EDTA pH 8, 1.4 M NaCl, 1% polyvinylpolypyrrolidone, 0.1% 2-mercaptoethanol) and shaken in a Mini-Beadbeater (Biospec Products) for 30 s at 5000 rpm. After mixing, samples were incubated at 65 C for 2 h. The DNA was purified in 24 : 1 chloroform : isoamyl alcohol and further purified to reduce PCR inhibitors by passing the extract over QIAAmp Spin Columns (QIAGEN Inc., Valencia, California) according to the manufacturer's instructions.

PCR was performed in 50 μL reactions (1× enzyme buffer, 200 μM dNTP, 0.4 μM of each PCR primer, 2.5 U RedTaq DNA polymerase (Sigma, St Louis, Missouri), and 1 μL template DNA). Genes that code for the SSU and ITS were amplified respectively with primer sets NS1-NS4 and ITS5-ITS4 (White et al 1990) and those that code for the LSU were amplified with LROR-LR5 (Vilgalys and Sun 1994). SSU reaction conditions were 35 cycles of denaturation at 94 C for 60 s, annealing at 52 C for 60 s and extension at 72 C for 60 s. LSU and ITS reaction conditions differed only by alteration of the annealing step to 50 C and 55 C, respectively. A negative control (no DNA) was included in each set of reactions. After amplification, PCR products were prepared for direct sequencing by precipitation in one-half volume of 9 M NH_4OAc and two volumes isopropyl alcohol. Cycle sequencing was performed with dye-terminator chemistry on an ABI model 377 fluorescent sequencer (PE Applied Biosystems Inc., Foster City, California) at the Central Services Laboratory of the Center for Gene Research and Biotechnology at Oregon State University. Primers NS1, NS4, NS3 (White et al 1990) and SR7 (R. Vilgalys, 5′-GTTCAACTACGAGCTTTTTAA-3′) were used to sequence both strands of SSU PCR products, LROR, LR3, and LR5 the LSU (Vilgalys and Sun 1994) and ITS5 and ITS4 for the ITS. Contigs were assembled and the overlapping sequences edited with the Staden package (Staden 1996). Gapped-BLAST (Altschul et al 1997) was used to check for contaminant sequences by comparison with GenBank accessions. Sequences identified by BLAST searches with high similarity to *P. nudus*, *P. gaeumannii* and *V. inaequalis* were downloaded and included in analyses.

Sequence alignment and phylogenetic analyses.—New SSU, LSU and ITS sequences determined in this study (TABLE I) were aligned with those obtained from nucleotide sequence libraries by means of the multiple alignment program MUSCLE v3.6 (Edgar 2004). Gaps and ambiguously aligned positions for each of the three datasets were eliminated with the software program Gblocks 0.91b with minimum block length set at 3 (Castresana 2000). The SSU and LSU datasets then were combined with missing sequence portions coded as ''N''.

Parsimony analysis was performed with PAUP* 4.0b10 (Swofford 2003). Maximum parsimony (MP) trees were inferred from heuristic searches with 1000 random sequence additions with the MULPARS and TBR options specified. Support for clade stability was estimated from 1000 nonparametric bootstrap replicates (Felsenstein 1985) using 5 random sequence additions with TBR branch swapping. In separate analyses we constrained parsimony searches of the combined SSU+LSU to address whether the data could support these phylogenetic hypotheses: (i) monophyletic *Phaeocryptopus*, (ii) *Phaeocryptopus gaeumanii* included in Venturiaceae (as delimited by parsimony and Bayesian analyses), (iii) *Phaeocryptopus nudus* included in Venturiaceae, (iv) *Rhizosphaera* species and *P. gaeumannii* monophyletic, (v) *Rhizosphaera* species and *P. nudus* monophyletic. Only the nodes of interest were resolved in the five constraint trees which were subjected to heuristic searches at the above settings in PAUP*. The most parsimonious constrained and unconstrained trees were compared by means of the Templeton (1983) and winning-sites tests in PAUP*.

Bayesian analysis was performed with MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001) with the following settings. The maximum likelihood model employed 6 substitution types ("nst = 6"), with base frequencies estimated from the data ("basefreq = estimate"). Rate variation across sites was modeled using a gamma distribution, with a proportion of sites being invariant (rates = "invgamma"). The Markov chain Monte Carlo search was run with 4 chains for 3 000 000 generations and trees were sampled every 100 generations. The program Tracer v1.3 (http://evolve.zoo.ox.ac.uk/software.html?id=tracer) was used to ensure that stationarity was achieved and determine the point of burn-in. The first 2000 trees were discarded as burn-in. Phylogenetic trees were visualized with the program TreeView (Page 1996). Clade support was considered strong with Bayesian posterior probabilities (PP) ≥ 0.95 and parsimony nonparametric bootstrap proportions (BS) $\geq$ 70% (Lutzoni et al 2004). Support was considered moderate with either PP ≥ 0.95 or BS $\geq$ 70%.

RESULTS

SSU and LSU rDNA.—Approximately 1100 base-pairs of SSU and 900 base-pairs of LSU rDNA were amplified from each of the isolates sequenced in this study. GenBank accession numbers of the 50 new sequences from 22 taxa are provided (TABLE I). Culture collection sequences of *P. gaeumannii*, *P. nudus*, *V. inaequalis* and *V. pyrina* each were identical to those obtained from cultures isolated independently in this study. We therefore assumed that these taxa were identified correctly and included the consensus sequence of each species in phylogenetic analyses. The initial SSU alignment with 50 sequences obtained from GenBank (TABLE I) included 1645 nucleotide positions and 73 taxa, including the Arthoniomycetes *Dendrographa leucophaea f. minor* and *Roccella fuciformis* as the designated outgroup. Gblocks selected 971 (59%) positions as conservative enough to satisfy assumptions of positional homology. The initial LSU alignment yielded a matrix 1125 positions, 768 (68%) of which were selected by Gblocks. The combined dataset consisted of 1739 characters. Of these 1246 were constant, 122 were variable but parsimony uninformative and 371 were parsimony informative, yielding 32 equally parsimonious trees (1618 steps, CI = 0.43, RI = 0.78, RC = 0.34). Results of the parsimony analysis were similar to the Bayesian analysis (FIG. 1). The mean log-likelihood of 56 000 trees from the two runs after stationarity was achieved was –11 201.

In all trees the two *Phaeocryptopus* species, *P. gaeumannii* and *P. nudus*, occurred in separate clades. The Templeton and winning sites tests rejected monophyly of the genus because heuristic searches on trees constraining the two taxa in the same clade always yielded trees that were at least 53 steps longer and always significantly worse than unconstrained trees ($P < 0.0001$). There was strong support (PP = 1.0, BS = 100%) for a monophyletic clade including *P. gaeumannii* with the four *Mycosphaeralla* species including lectotype *M. punctiformis*. Also included in this clade are two species of *Rasutoria* (Euantennariaceae). There was strong support for a Capnodiales clade (PP = 0.97, BS = 98%) that included members of the Capnodiaceae, Euantennariaceae and Mycosphaerellaceae and a strongly supported clade that included *Sthughesia juniperi*, *Xenomeris raetica* and an unknown species of *Stomiopeltis*.

Phaeocryptopus nudus, type of the genus, was moderately supported (PP = –0.94, BS = 91%) as belonging to an unresolved polytomy including *Rhizosphaera* form-species and *Plowrightia abietis* (Barr) Barr in the Dothioraceae. These sequences were similar and branch lengths among them short. The Templeton and winning sites tests rejected monophyly of *Rhizosphaera* spp. and *P. gaeumannii* ($P < 0.0001$) but could not reject monophyly of *Rhizosphaera* spp. and *P. nudus* (Templeton, $0.0606 \leq P \leq 0.1282$; winning sites, $0.0636 \leq P \leq 0.1849$).

Neither *P. nudus* nor *P. gaeumannii* grouped with core members of Venturiaceae, and both Templeton and winning sites tests rejected their placement in Venturiaceae ($P < 0.0001$). Whereas placement of Venturiaceae was unresolved, strong support (PP = 1.0, BS = 100%) for a monophyletic Venturiaceae included the three species of *Venturia*, *Protoventuria barriae*, *Metacoleroa dickiei*, *Apiosporina collinsii*, and *Dibotryon morbosum* as expected. Also included in the Venturiaceae clade was *Tyrannosorus pinicola*, a dothideomycete with uncertain affiliation. *Platychora ulmi*,

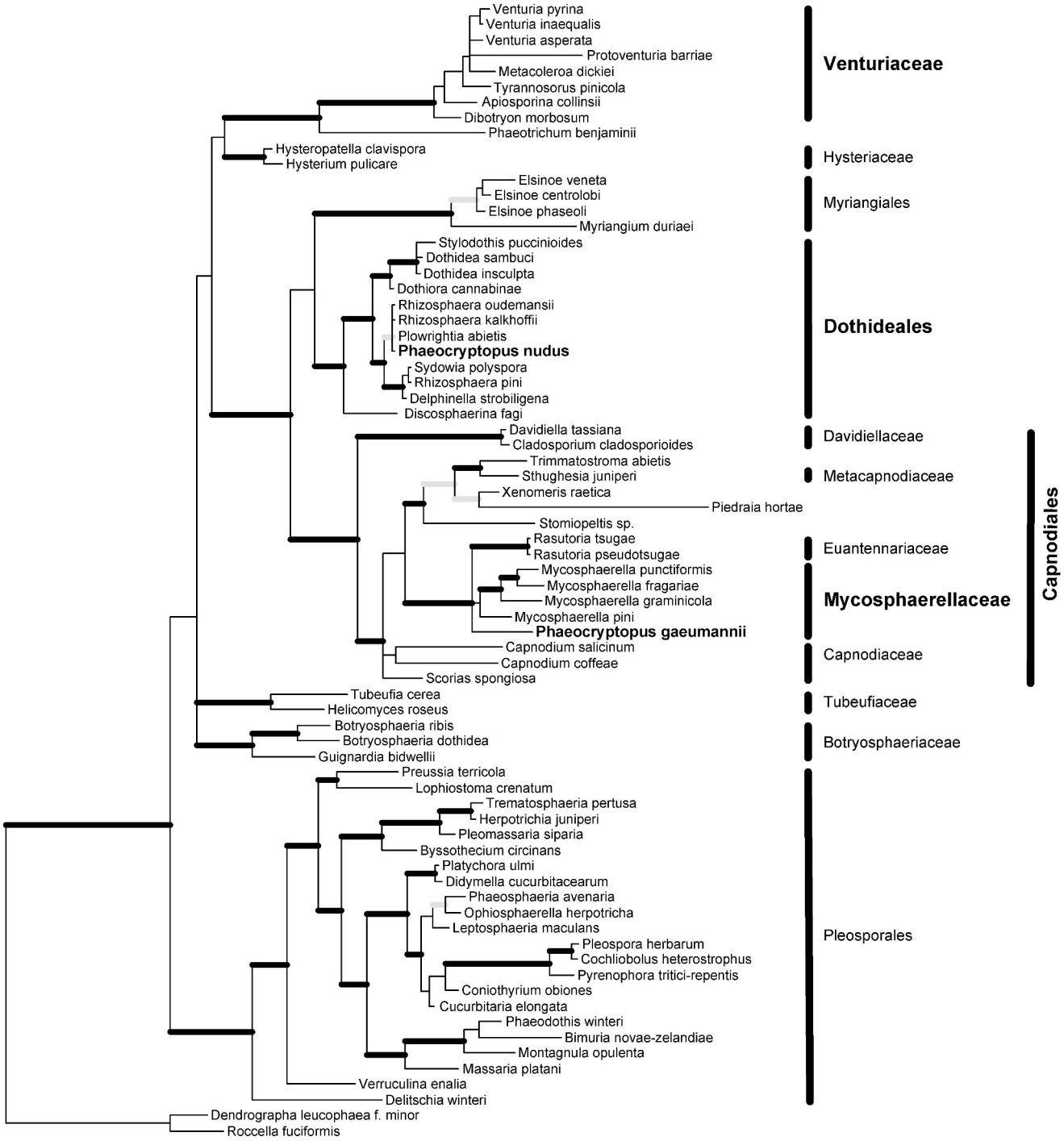

FIG. 1. Estimated dothideomycete phylogeny. Bayesian 50% majority-rule consensus of 56 000 MCMCMC trees based on combined SSU and LSU rDNA sequences. Branches with posterior probabilities ≥ 0.95 and maximum parsimony bootstrap ≥ 70% are indicated with bold black lines. Bold gray lines represent branches with only one of the values above the threshold.

currently assigned to Venturiaceae, grouped with strong support (PP = 1.0, BS = 100%) with *Didymella cucurbitacearum* (uncertain classification) within a strongly supported clade of Pleosporales (PP = 1.0, BS = 100%).

ITS rDNA.—Of the 612 bp multiple sequence alignment, Gblocks selected 349 positions (57%) as conservative enough to satisfy positional homology. Of these characters 199 were constant, 40 were variable but parsimony-uninformative and 110 were

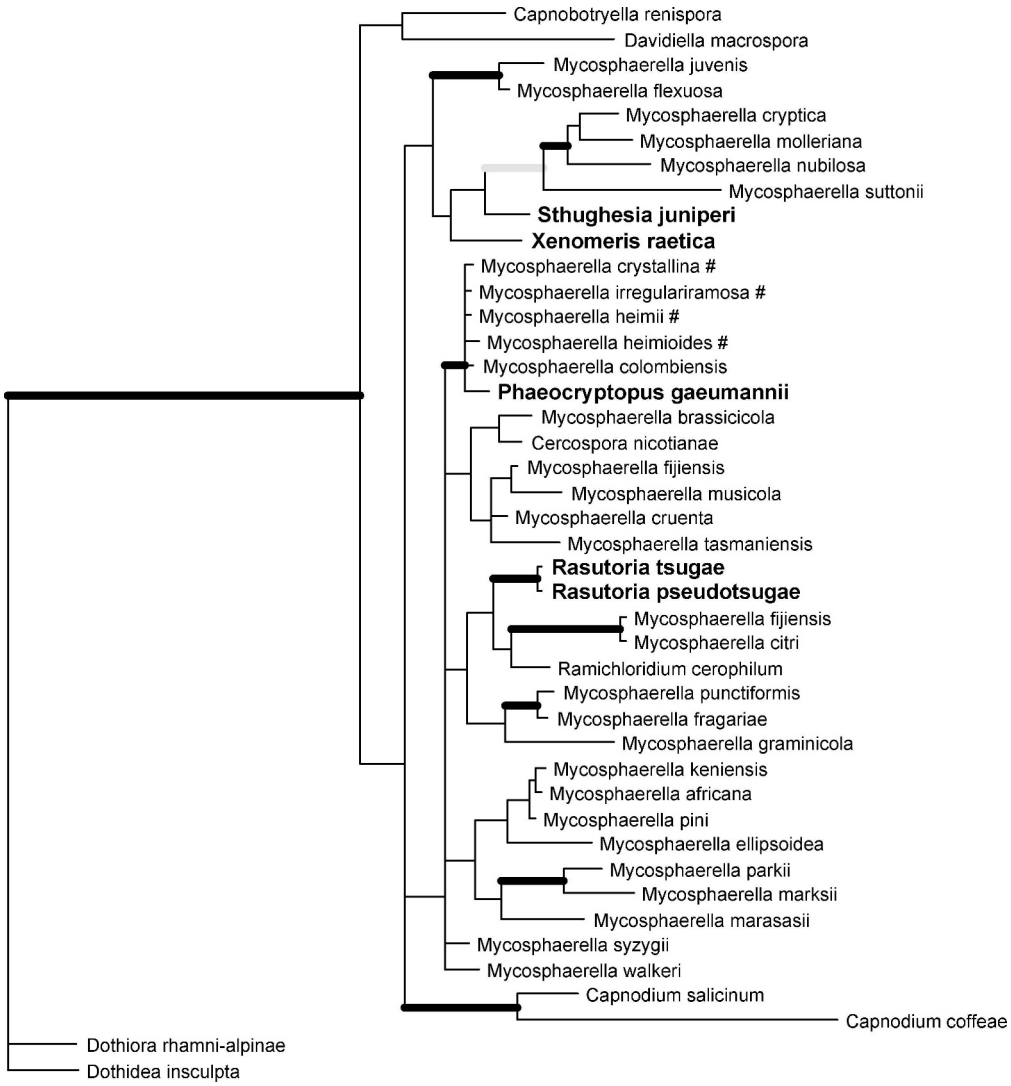

FIG. 2. Estimated *Mycosphaerella* phylogeny. Bayesian 50% majority-rule consensus of 56 000 MCMCMC trees based on ITS rDNA sequences. Branches with posterior probabilities ≥ 0.95 and maximum parsimony bootstrap ≥ 70% are indicated with bold black lines. Bold gray lines represent branches with only one of the values above the threshold.

parsimony-informative. Two MPTs were obtained (tree length = 518, CI = 0 47, RI = 0.5996). The mean log-likelihood of the 56 000 Bayesian trees sampled after burn-in was –2973. ITS sequences of both *P. gaeumannii* isolates were identical, therefore a consensus sequence was used for analyses. *P. gaeumannii* formed a strongly supported (PP = 0.98, BS = 79%) monophyletic clade with *M. crystallina*, *M. irregularimosa*, *M. heimii*, *M. heimioides*, and *M. colombiensis* (FIG. 2). ITS sequences of the two *Rasutoria* species, *R. pseudotsugae* and *R. tsugae*, were identical. The two *Rasutoria* species, *Sthughesia juniperi*, *Xenomeris raetica*, and *P. gaeumannii* all were supported strongly within the *Mycosphaerella* ingroup (PP = 1.00, BS = 100%).

DISCUSSION

Phylogenetic analyses of nuclear rDNA sequence data provided significant and robust evidence that *Phaeocryptopus gaeumannii* is not congeneric with the genus type *P. nudus*. Instead the analysis of combined small and large subunit ribosomal DNA indicated that closest relatives are species in Mycosphaerellaceae, which grouped within Capnodiales. Capnodiales comprise a group known as the ''sooty molds'' because of profuse superficial hyphal development. While original descriptions of *P. gaeumannii* did not note the presence of external mycelium, recent evidence has suggested otherwise (Stone et al 2007, Stone and Carroll 1985). But there are indications that some mycologists recognized this relationship

early in the taxonomic history of the fungus. When Theissen (1914) transferred *Asterina nuda* to the new genus *Adelopus*, he placed it in Capnodiaceae. However in our analyses *P. nudus* appears more closely related to species in Dothioraceae and Dothideaceae. It is unclear what material was examined in Theissen's and many other taxonomic revisions based on the type material. Indeed there has been much documented confusion over which of the two loculoascomycetous fungi present in Peck's type specimen packet that he and several following mycologists actually described (see Rohde 1936, Petrak 1938, Hahn 1947).

An additional complication arose when Petrak (1938), in an influential revision, synonymized *Adelopus* and *Phaeocryptopus* but without examining the types for either. Petrak merged the two genera in spite of the reported presence of pseudoparaphyses in *Phaeocryptopus* (Naumov 1915) that are absent in *Adelopus* (Hahn 1947). Because descriptions of the two genera differ with respect to ascospore pigmentation and hamathecial elements, Hahn (1947) remained skeptical of this treatment, although it has been generally accepted. Hahn (1947) stated that he was unable to obtain the holotype of *P. abietis*. The Naumov holotype collection of *P. abietis* since has been located and examined and compared to the type collection of *Asterina nuda* Peck including a redescription and illustrations (Shoemaker 1965). Comparison of the holotype collections of *Phaeocryptopus abietis* Naumov and *P. nudus* (Peck) Petrak (≡*Aster-Asterina nuda* Peck) revealed that they are morphologically indistinguishable and the name *P. abietis* is a synonym of *P. nudus*. Comparative morphological studies of *P. nudus* and *P. gaeumannii* will be reported later. The Naumov holotype material contains several fungi, noted on the specimen label, but *P. abietis* is clearly distinguishable. Pseudoparaphyses or interthecial threads (shown in an illustration in Naumov (1915)) are absent, and ascospores within asci are hyaline. The structure in the original Naumov (1915) illustration might be a filiform ascospore of an unrelated fungus.

The early literature also suggests that species of *Rhizosphaera* are the anamorphs of *Phaeocryptopus* because of morphological similarities between the fruiting structures and frequent cohabitation (Petrak 1925, 1938). However developmental studies have never confirmed this view (but see Stone and Carroll 1985 associating phialide-like cells with the occurrence of *P. gaeumannii*). The putative connection was a matter of dispute between Rohde (1937) and Petrak (1925, 1938). Detailed comparisons of developmental and culture characteristics between *P. nudus*, *P. gaeumannii* and two *Rhizosphaera* species (Rohde 1937) provided empirical evidence against the connection. In particular Rohde (1937) noted that the mycelia developing from single ascospore isolates of *P. gaeumannii*, which never formed conidia in vitro, could not be homologous to that of a *Rhizosphaera* species found in frequent association with *P. gaeumannii* that always produced conidia in culture. Petrak (1938) however dismissed Rohde's observations and again asserted a connection between *Rhizosphaera* and *Phaeocryptopus* based on morphological similarities. Kobayashi (1967) was unable to verify the connection between *R. pini* and *P. nudus* but reported that in culture *P. nudus* was similar to an undescribed *Rhizosphaera* species and also noted that cultures of *R. kalkhoffii* were different from *P. gaeumannii*, as reported by Rohde (1937). Despite this uncertainty, Petrak's opinion has prevailed. *Rhizosphaera* was accepted as anamorphic *Phaeocryptopus* in the 8th edition of the Dictionary of the Fungi (Hawksworth et al 1995).

Phylogenetic analyses reported in this paper now might help to resolve this matter. The SSU+LSU analyses confirm Rohde's (1937) finding that *P. gaeumannii* and *Rhizosphaera* are unrelated. At the same time our results support a close relationship between *P. nudus* and the three *Rhizosphaera* species examined. Explicit tests could not reject monophyly of *P. nudus* and the three *Rhizosphaera* species. Sequences of all four species were similar at both loci, displaying only two adjacent polymorphic regions in the SSU and two distant regions in the LSU. *Rhizosphaera* species produce *Hormonema* synanamorphs in culture (Funk 1985, Butin and Kehr 2000), and *Hormonema* is well established as anamorphic Dothioraceae (de Hoog et al 1999), in agreement with the placement of *Rhizosphaera* species in the SSU+LSU analyses. Of interest, sequences of *Plowrightia* (=*Xenomeris*) *abietis*, another *Abies* parasite but growing on twigs, also were similar to the *P. nudus* and *Rhizosphaera* sequences and were identical to *R. oudemansii*. *Plowrightia* also produces a *Hormonema* anamorph in culture (Funk 1981, Hermanides-Nijhof 1977) also in agreement with its placement in the SSU+LSU analyses as a member of Dothidiales and separate from *X. raetica*, currently classified in Venturiaceae, but grouping with Capnodiales in our analysis.

Regardless of the taxonomic and nomenclatural difficulties, neither of the fungi included in this study presently assigned to the genus *Phaeocryptopus* belong to Venturiaceae. The fungus that causes Swiss needle cast, identified here as *P. gaeumannii*, is placed unambiguously as a member of the Capnodiales based on SSU+LSU analysis. Based on these and two additional loci, Schoch et al (2006) provided evidence

for a monophyletic Mycosphaerellaceae in Capnodiales. In our analysis *P. gaeumannii* groups unambiguously within this family. Morphological evidence, such as the lack of a hamathecium and the presence of superficial mycelium, further supports this placement. On the other hand *P. nudus* is much more closely allied with Dothideales, probably in Dothioraceae, although a more extensive sampling of other members of the family, particularly the type, would augment this argument considerably.

The ITS analysis indicates that *P. gaeumannii*, together with the two *Rasutoria* species examined here, *R. pseudotsugae* and *R. tsugae*, are incorrectly classified in separate genera and also group within *Mycosphaerella*. *Phaeocryptopus* and *Rasutoria* both are characterized by having separate, globose, thin-walled pseudothecia, ovoid to cylindrical, bitunicate, eight-spored asci, no pseudoparaphyses, and hyaline to pale brown, equatorially euseptate, fusoid to obovate ascospores. These characters also are shared by *Mycosphaerella*. *Rasutoria* was introduced by Barr (1987a) for *Dimerosporium abietis* Dearness, characterized as being hypophyllous on conifer needles and having setose ascocarps, superficial on a radiating mycelium that penetrates at the stomata. *Phaeocryptopus gaeumannii* ascocarps are smooth, emerging from Douglas-fir needle stomata and remaining attached to internal, intercellular mycelia by a cluster of basal hyphae in the substomatal chamber. A superficial, radiating mycelium also emerges from developing ascocarps, spreading across the needle surface and reentering the needle through unoccupied stomata (Stone and Carroll 1985, Stone et al 2007).

Barr (1987a) proposed *Sthughesia* typified by *St. juniperi* (≡*Xenomeris juniperi* [Dearn.] M.E. Barr & E. Müll. ≡*Dimerium juniperi* Dearn.) in Metacapnodiaceae of Capnodiales. Eriksson and Hawksworth (1988) considered *Sthughesia* to be a probable but questionable synonym of *Metacapnodium* Speg. In most current classifications *Xenomeris* is placed in Venturiaceae of Pleosporales (e.g. Eriksson 2006). With the proviso that we do not have data from the type species of *Metacapnodium* or *Xenomeris* Sydow, we found in the SSU+LSU analysis that *Sthughesia juniperi* occurred in a sister relationship with *Trimmatostoma abietis*, a dematiaceous, hyphomycetous anamorph of uncertain affiliation, and separate from *Xenomeris raetica* (E. Müll.) Petr. on a node with moderate support (FIG. 1). The placement of *Sthughesia* in the LSU/SSU analysis appears to support Barr's (1987a) opinion that *X. raetica* and *St. juniperi* are not congeneric, as well as her placement of the genus in Capnodiales. The two species also grouped together in the ITS analysis, however with no support. *Stomiopeltis* sp. of Micropeltidaceae with uncertain ordinal placement (Eriksson 2006), *St. juniperi* and *X. raetica* in the SSU+LSU analyses presented here were all in a sister group of Mycosphaerellaceae within Capnodiales. In the ITS analysis, *St. juniperi* and *X. raetica* also grouped together within *Mycosphaerella*. Thus the combination of morphological characters represented by species found to group within *Mycosphaerella* suggests that the genus is much more diverse than hitherto recognized. The LSU analysis of Batzer et al (2005) also grouped *Stomiopeltis* spp. within a clade of *Mycosphaerella* spp. and *Mycosphaerella* anamorphs. Transfer of all these species to *Mycosphaerella* is anticipated but will entail some modification of the genus to incorporate these characters and so will be considered later.

In the ITS analysis *P. gaeumannii* occurred in a clade with several pathogenic *Mycosphaerella* species known to produce red crystals in culture (Crous et al 2000, Crous 2001). This well supported clade also grouped near other species which produce diffusing reddish pigments that have been connected to the polyketide phytotoxins cercosporin and dothistromin (Goodwin et al 2001, Bradshaw 2004). These include the cercosporin-producing *Cercospora nicotianae* and *Mycosphaerella pini*, another conifer foliar pathogen that produces the similar phytotoxin dothistromin (Bradshaw 2004) (FIG. 2). Isolates of *P. gaeumannii* also have been observed to produce diffusing red pigments in culture (Winton and Stone unpubl.). In view of the phylogenetic placement of *P. gaeumannii* within this group, the possibility that this pigment might be a cercosporin-like substance that might play a role in the pathology of Swiss needle cast disease must be considered.

This study has added to emerging concepts of higher level loculoascomycete systematics for several groups. Venturiaceae, as presently defined (i.e. Kirk et al 2001), is polyphyletic. Kruys et al (2006) presented evidence for a monophyletic Venturiaceae, including two species, *V. inaequalis* and *Metacoleroa dickiei*, also included in our analysis. Five additional species sequenced in this study, *Apiosporina collinsii*, *Dibotryon morbosum*, *Protoventuria barriae*, *V. asperata* and *V. pyrina*, in addition to *Tyrannosorus pinicola* (from Schoch et al 2006) formed a strongly supported monophyletic group. However the ordinal placement of these core members of the family remains uncertain, grouping consistently with neither Pleosporales nor Dothideales. Ordinal placement of Venturiaceae also was unresolved in Kruys et al (2006). Furthermore several genera that have been classified traditionally in Venturiaceae, *Phaeocryptopus*, *Platychora* and *Xenomeris* clearly are not related to one another or to the core Venturiaceae clade and must be assigned elsewhere. The generic placement of *Dibotryon morbosum* (Schwein.) Theiss. & Syd. has been somewhat

controversial. Von Arx transferred the species to *Apiosporina* (von Arx 1954). However Barr (1989) recommended that *D. morbosum* be retained in *Dibotryon,* based on superficial hyphae and ascoma shape. Based on the SSU+LSU analysis, separate generic placements of *A. collinsii* and *D. morbosum* appear to be justified. The sequences for the two species are more dissimilar than e.g. *Venturia* spp. Both species grouped in a strongly supported Venturiaceae clade.

Schoch et al (2006) provide multigene sequence evidence for recognition of Davidiellaceae and Mycosphaerellaceae as distinct families within Capnodiales, and our SSU+LSU analysis also supports this finding. There was strong support for Mycosphaerellaceae, including four *Mycosphaerella* species, *P. gaeumannii,* the two *Rasutoria* species, and Davidiellaceae, comprising *Davidiella tassiana* and *Cladosporium cladosporioides*. Whether a separate Euantennariaceae, as in classifications of Barr (1987b) and Luttrell (1951) in the Capnodiales should be retained is unclear and should be revisited with additional sampling of genera within the family. The results of this study also agree with those of Schoch et al (2006) that Dothioraceae and Dothideaceae form a monophyletic Dothideales which is a sister group of the Capnodiales. While this arrangement is supported by morphological characters, such as the absence of hamathecial tissue in both orders, inclusion of the species that are types for genera on which higher categories are based in future analyses would considerably bolster this placement.

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